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TITLE: A Novel EphA4-Based Small Molecule-Based Therapeutic Strategy for Prevention and Treatment of Post-Traumatic Osteoarthritis

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14. ABSTRACT Post-traumatic osteoarthritis (PTOA) is a painful and debilitating disease of the joints, characterized by intra-articular inflammation, deterioration of articular cartilage, and degenerative changes to peri-articular and subchondral bone. There is currently no effective therapy. This project capitalizes our discovery that the forward signaling of EphA4 receptor has both an anti-catabolic effect (on osteoclasts/chondroclasts) and an anabolic effect (on chondrocytes) on skeletal tissues and seeks to develop a small molecule-based therapy for PTOA that involves merely direct injection of a soluble EphA4-acting EfnA-fc protein into the injured synovium. We will first confirm that EfnA-fc-mediated activation of the forward signaling of EphA4 in synoviocytes and infiltrating neutrophils/monocytes would reduce survival and the release of pro-inflammatory cytokines and MMPs, but, in articular chondrocytes, would enhance chondrogenesis <i>in vitro</i> . We will then optimize a small molecule EfnA-fc-based strategy involving injections of an EphA4-binding EfnA-fc into the joint by determining an appropriate EfnA-fc ligand for EphA4, an optimal dosage, and the time course of the effect, and an appropriate duration between injections. Finally, we will determine whether the small molecule EfnA-fc-based therapeutic strategy can prevent PTOA-mediated degradation of articular cartilage in early PTOA, and also can promote regeneration of articular cartilage in established PTOA. If this therapy is effective, it will improve the quality of life of PTOA patients, not only in the active duty military personnel and the veteran population, but also in the civilian population. It may even allow the warrior to return to active duty. This proposal has high military benefits.					
15. SUBJECT TERMS Osteoarthritis; post-traumatic osteoarthritis; synoviocytes; articular cartilage regeneration; intra-articular fractures; chondrocytes; articular chondrocytes; articular cartilage degradation.					
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1. INTRODUCTION:

Post-traumatic osteoarthritis (PTOA) is a painful and debilitating disease of the joints, characterized by intra-articular inflammation, deterioration of articular cartilage, and degenerative changes to peri-articular and subchondral bone. Currently, there is no effective therapy that would prevent or treat PTOA. This project capitalizes our discovery that the forward signaling of EphA4 receptor has an anti-catabolic effect on osteoclasts/chondroclasts but an anabolic effect on chondrocytes on skeletal tissues and seeks to develop a small molecule-based therapy for PTOA that involves merely direct injection of a soluble EphA4-acting EfnA-fc protein into the injured synovium. This therapy on the one hand should activate the forward signaling of EphA4 in infiltrating monocytes and in osteoclasts/chondroclasts to suppress the release of pro-inflammatory cytokines and proteases to degrade articular cartilage, and it on the other hand should also act on articular chondrocytes to promote regeneration of the damaged articular cartilage. In this project, we will first confirm that EfnA-fc-mediated activation of the forward signaling of EphA4 in synoviocytes and infiltrating neutrophils/monocytes would reduce survival and the release of pro-inflammatory cytokines and MMPs, but, in articular chondrocytes, would enhance chondrogenesis *in vitro*. We will develop a mouse intra-articular fracture model of PTOA for use in this study. We will then optimize a small molecule EfnA-fc-based strategy involving injections of an EphA4-binding EfnA-fc into the joint by determining an appropriate EfnA-fc ligand for EphA4, an optimal dosage, and the time course of the effect, and an appropriate duration between injections. Finally, we will determine whether the small molecule EfnA-fc-based therapeutic strategy can prevent PTOA-mediated degradation of articular cartilage in early PTOA, and also can promote regeneration of articular cartilage in established PTOA. If this therapy is shown to be effective, it will improve the quality of life of PTOA patients, not only in the active duty military personnel and the veteran population, but also in the civilian population. It may even allow the warrior to return to active duty.

2. KEYWORDS:

Osteoarthritis; post-traumatic osteoarthritis; EphA4 receptor; regenerative therapy; synoviocytes; articular cartilage regeneration; intra-articular fractures; chondrocytes; articular chondrocytes; articular cartilage degradation.

3. ACCOMPLISHMENTS:

This project contains the following three approved Study Aims and three sets of Technical Tasks.

3.1. Study Aims:

- Aim 1 - To show that activation of EphA4 in synovial monocytes reduces release of pro-inflammatory cytokines and proteinases, but in articular chondrocytes enhances chondrogenesis *in vitro*.

- Aim 2 - To develop and optimize the EfnA-fc-based strategy involving intra-articular injection of an EfnA-fc into PTOA joint of a mouse.
- Aim 3 - To establish that the EfnA-fc-based therapy is effective in preventing PTOA development and/or treating advanced PTOA in mice.

3.2. Tasks/Approach:

- Task 1 – Demonstrate in vitro efficacy of the EfnA4-fc approach.
 - Subtask 1.1 – Isolate monocytes and articular chondrocytes for testing.
 - Subtask 1.2 – Show that EfnA4-fc inhibits activity of infiltrating monocytes.
 - Subtask 1.3 – Show that EfnA4-fc enhances activity of articular chondrocytes.
- Task 2 – Development and optimization of EfnA-fc-based therapy for PTOA.
 - Subtask 2.1 – Identify appropriate form and dosages of EfnA-fc protein for use.
 - Subtask 2.2 – Determine the time-dependent effects of the therapy.
 - Subtask 2.3 – Compare efficacy of single vs. multiple EfnA-fc administration.
- Task 3 – Establish therapeutic efficacy of the EfnA-fc-based strategy.
 - Subtask 3.1 – Show therapeutic efficacy on prevention of PTOA development.
 - Subtask 3.2 – Show therapeutic efficacy on treatment of established PTOA.

During the first year of this project (the reporting period), we focused on three aspects of the work: 1) to obtain necessary regulatory approval and to recruit appropriate personnel prior to the initiation of the project; 2) to complete the proposed work in Task 1; and 3) to establish the intra-articular fracture model of PTOA, which is required for the proposed work in Task 2 and Task 3.

3.3. Accomplishments made during the reporting period (15-08-2014 to 14-09-2015):

3.3.1 Summary of Accomplishments:

- We successfully established a method to isolate synovial fibroblasts from knee joints of C57BL/6J mice (28-02-2015).
- We successfully established a method to isolate and expand chondrocytes from articular cartilage of knee joints of C57BL/6J mice (28-02-2015).
- We have demonstrated that activation of the forward signaling of cartilage producing ability (30-05-2015).
- We showed that the EfnA4-fc-mediated enhancement of chondrocyte maturation appeared to be specific for articular chondrocytes and growth plate chondrocytes, but not sternum-derived chondrocytes (30-05-2015).
- We showed that treatment of chondrocytes with EphA4-fc had no significant effects on chondrocyte maturation, suggesting that either chondrocytes do not express substantial amounts of EphA4-interacting Efn's or activation of the reverse signaling of the EfnA4 pathway had no effect on chondrocyte maturation (30-05-2015).
- We showed that treatment with articular chondrocytes with EfnhA4-fc protein to activate the EphA4 forward signaling did not affect their proliferation or apoptosis (30-09-2015).
- We have developed the murine intra-articular fracture model as a reliable post-traumatic osteoarthritis (30-09-2015).

- We showed that significant post-traumatic osteoarthritis was reliably developed in mice approximately 8 weeks after subjected to an intra-articular fracture (30-09-2015)

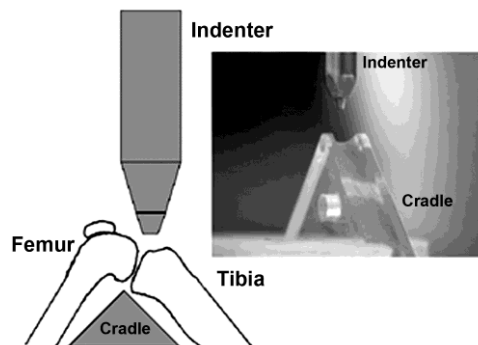
3.3.2. Detailed Description of Accomplishments:

a. To obtain the necessary regulatory approval and to recruit a qualified research technician to assist the research team to perform the proposed experiments.

During the first three months of the project, we successfully obtained approval for our Animal Component of Research Project (ACORP) from our local IACUC and approval for our BioSafety Papers from our IBC IACUC in April 2014. An amendment of an ACORP was later submitted to IACUC to correct an error on the total number of animals. The approved ACORP and the amendment were submitted to ACURO for approval and final approval from the ACURO was secured on July 14, 2014. Funding for this work was released on August 15, 2014.

With respect to recruitment of research technician for this project, advertisements were placed in local newspapers and relevant Websites in September 2014. We received more than 50 applications. We selected 10 applications for telephone interviews in October 2014, and invited four candidates for face-to-face interview in late October 2014. An offer was eventually made to and was accepted by Ms. Amandeep Kaur. Ms. Kaur is a recent graduate from the California State University at San Bernardino with a B.S. degree in Biology. Prior to joining our group, she worked in a commercial research laboratory for about one year. She started the position in late November 2014.

b. Task 1: To determine whether the EfnA4-mediated activation of the forward signaling of EphA4 in synoviocytes and infiltrating monocytes/macrophages would reduce cell survival and the release of pro-inflammatory cytokines and MMPs, but in the articular chondrocytes, would enhance chondrogenesis and their survival in vitro.



*Figure 1: The intra-articular tibial plateau fracture model of PTOA. The protocol was adapted from Furman *et al.* (J Orthop Res 25:578, 2007). The knee joint is bended and placed on a supporting bar. An indenter is placed on the tibial plateau. A mechanical force is applied through an indenter using an Instron mechanical tester to create partial intra-articular fractures.*

There are three subtasks in Task 2, which are scheduled to be completed from month 3 to month 15 of the project.

c. Subtask 1.1: To isolate synoviocytes, infiltrating monocytes/macrophages, and articular chondrocytes from the injured synovium of C57BL/6J mice one week after the intra-articular fracture.

To produce PTOA-like symptoms in the knee, eight week-old C57BL/6 mice undergo an impact to the tibial plateau. The injury was produced using an “Instron” servohydraulic tester with the animal under inhalation anesthesia (3% isoflurane, 0.5 L/min oxygen delivered through a Bains circuit). The right knee of each mouse was positioned on an apparatus attached to the load cell of an Instron servohydraulic tester (Fig. 1). To injure the articular cartilage at the tibial plateau, the excursion of

an indenter blade was set to a point 1.23 mm about the support on which the knee joint was positioned. Impact was computer-controlled using Instron “FastTrack 2” software. This approach produced a force of approximately 60 to 80 N at the tibial plateau. Analgesia was administered immediately after the procedure (50 µg/kg buprenorphine, subcutaneous), and the animals observed for two days afterward for signs of pain or discomfort. X-ray analysis of the knee immediately after the procedure revealed no fractures to the bone of the femoral condyle or to the tibial plateau, suggesting that the force was absorbed by the articular cartilage of the tibia.

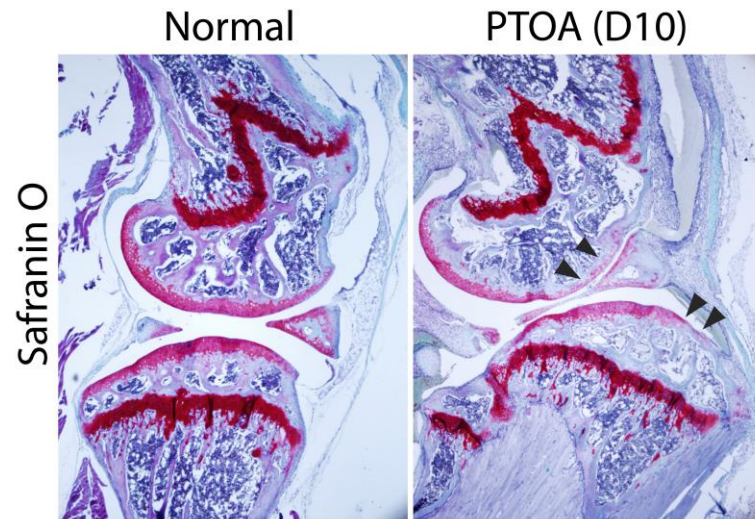


Figure 2: Comparison of the injured (right panel) versus contralateral control articular cartilages 10 days post surgery by Safranin-orange-stained staining. The OA cartilage (Arrowhead) shows the thinner layer of articular cartilage with less intensity.

The animal was allowed free movement at all times. No evidence for impaired mobility was observed. Harvest was performed at one week post-procedure, when the initial acute inflammation subsides and when chronic inflammation and motion of the joint should initiate the development of PTOA, which was consistently developed at ~10 days post-trauma (Fig. 2). Moreover, PTOA is developed regardless of intra-articular fractures are produced or not. We prefer to not have actually produced fractures, since fracture healings can confound the evaluation of osteoarthritis development.

For isolation of synoviocytes, mice were sacrificed to obtain the synovial pad. The synovial pad was isolated from the posterior aspect of the knee, and digested in 0.1% collagenase IV, 0.005% DNase I in incomplete media for 2 to 4 hours. The undigested debris was removed by filtration of the digest through a sterile 40 µm mesh. The cells were washed and resuspended in 1 ml complete DMEM/F12 media [15% fetal bovine serum (FBS) with penicillin-streptomycin and 1% amphotericin B] and cultured at 37°C under an atmosphere of 5% CO₂. Fibroblast-like colonies were visible between one and two weeks of culture, and were frozen for preservation and expanded in further culture for in vitro studies. However, the overall yield of synoviocytes from each single mouse, even after two weeks of culturing, was relatively low, due to the fact that the amounts of synovial tissues isolated from each mouse were rather limited. Accordingly, we may have to pool isolated cells from several mice in order to have sufficient numbers of cells for the proposed studies.

An examination of the literature reveals that expression of the genes S100a4 and smooth muscle actin (SMA) is characteristic of synovial fibroblasts. Our initial measurements of gene expression by real-time RT-PCR have established that the conditions for most efficient amplification of these genes as compared to the housekeeping gene (PP1A, cyclophilin A), confirming that these isolated cells are indeed synoviocytes.

To establish a method for isolation of articular chondrocytes from the knees of C57BL/6J mice, 6-8 weeks old C57BL/6J mice were sacrificed and articular cartilages from both knees were collected, rinsed in PBS, and placed into 15 mL centrifuge tubes containing approximately 3 mL of 2.5% trypsin [PBS, 0.85% saline, Atlanta Biologicals] on ice for an hour. Collagenase II [220 U/mg] and Pronase [2 mg/mL] were dissolved in Minimal Essential Medium alpha Medium (α -MEM) containing penicillin and streptomycin. Digestion was carried out in a shaking water bath at 37 °C for 1.5 hours and terminated by adding 10 mL of α -MEM supplemented with 5% FBS into the digestion mixture. This would remove cells from non-articular cartilage tissue and expose articular cartilage for articular chondrocyte isolation. The articular cartilage was then digested with Collagenase P overnight. The samples were then washed with medium containing 2% FBS, and were then digested with freshly prepared Collagenase II for an additional 1.5 hours. Once the digestion was complete, samples were washed and further digested with Collagenase II overnight. Following overnight incubation, the digestion was terminated by adding α -MEM containing 10% FBS. The released cells were filtered through a 70- μ m sterile filter. The released articular chondrocytes were then collected by centrifugation and seeded in α -MEM supplemented with 10% FBS. To confirm that the isolated cells were indeed articular chondrocytes, we measured the relative expression levels of type II collagen (a marker gene for proliferative chondrocytes), type X collagen (a marker gene for hypertrophic chondrocytes), and type I collagen (a marker for fibroblasts and osteoblasts) in these cells by real-time RT-PCR. We found that these cells expressed predominantly type II collagen with low levels of type X collagen mRNA. To confirm that these cells are articular chondrocytes, we cytochemically stained cells for acidic proteoglycan with Alcian blue after the cells were cultured for one week in α -MEM containing 5% FBS. As shown in Fig. 3, the cells showed morphology consistent with articular chondrocytes, containing proliferating chondrocytes without undergoing hypertrophy. More importantly, the cells stained strongly for Alcian blue, supporting their ability to produce cartilage tissues. Based on these findings, we conclude that we have established a method to effectively isolate articular chondrocytes from knee joints.

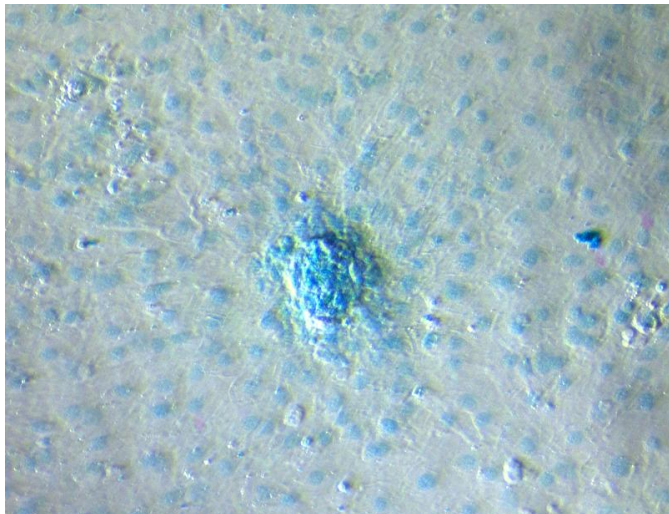


Figure 3. Isolated cultures of articular chondrocytes from knee joints of C57BL/6J mice stained with Alcian blue for acidic proteoglycan.

d. Subtask 1.2: To determine whether EfnA4-fc treatment of monocytes/macrophages and inflamed synovial fibroblasts would reduce release of pro-inflammatory cytokines and MMPs as well as reduce cell survival in vitro. During the several months, we have initiated work to isolate synovial monocytes and inflamed synovial fibroblasts from the injured knee joint. Unfortunately, the number of isolated synovial fibroblasts from a single inflamed knee joint was limited, even after culturing for two weeks. As a result, while we have established a method to isolate synoviocytes from the injured knee, we have not yet been able to isolate sufficient number of inflamed synoviocytes for the proposed work. We are working on two strategies: first, we are

optimizing the culturing conditions to accelerate growth of the synoviocytes in vitro; second, we plan to pool cells from multiple inflamed joints. Once sufficient numbers of cells are collected, we will initiate the proposed work. Consequently, subtask 1.2 is about 10% accomplished. This work will be continued during the second year of the funding.

e. Subtask 1.3: To determine whether EfnA4-fc treatment of articular chondrocytes would promote chondrocyte proliferation and maturation, as well as reduce cell survival in vitro. We next sought to evaluate whether activation of EphA4 forward signaling in the articular chondrocytes would promote their maturation and differentiation in vitro. Chondrocyte maturation and differentiation were assessed by their ability to form colonies of cartilage-forming cells after 7 days of culture, which was measured by their ability to produce acidic proteoglycan (indicated by the Alcian blue stain). To activate the forward signaling of the EphA4 signaling, we treated isolated articular chondrocytes with 10 ng/mL EfnA4-fc fusion protein (a soluble ligand of the EphA4 receptor). The acidic proteoglycan-producing colonies (as an index of cartilage formation) were identified with Alcian blue staining. Very briefly, 20,000 cells/well were added to a 24 well plate with α -MEM containing 10% FBS. Once cells approached confluence, they were treated with ephrinA4 (EfnA4)-fc chimera fusion protein or solvent vehicle for 7 days. Cells were then washed with TBS [2 mM CaCl_2] and fixed in 4% formaldehyde for 30 minutes at 4°C. TBS was used to rinse the wells again to remove excess

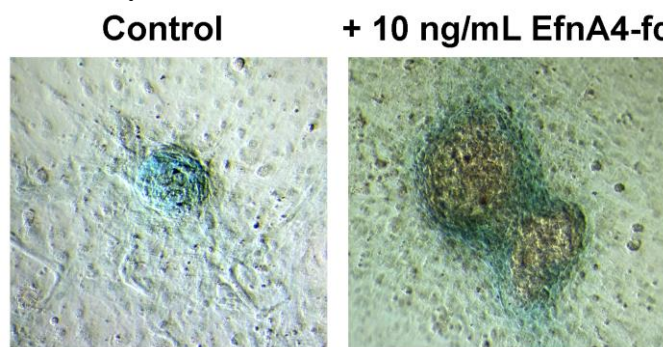


Figure 4. Alcian blue staining of representative colonies of acidic proteoglycan-producing cells derived from control articular chondrocytes (left) or from EfnA4-fc (10 ng/mL)-treated articular chondrocytes for 7 days.

media and formaldehyde. The cells were then stained with 1% Alcian Blue prepared in 3% acetic acid overnight. The next day, the plates were rinsed once with 3% acetic acid and once with distilled water. The distilled water wash was left to soak for two minutes to neutralize the remaining acid. We used a light microscope to capture images for analysis on ImagePro Plus 4.5 software [Media Cybernetics] to determine the area of each colony. Fig. 4 shows a representative acid proteoglycan-producing colony in control culture or EfnA4-fc-treated culture.

To further evaluate the effects of activation of the EphA4 forward signaling pathway in articular chondrocytes, we compared the effects of the EfnA4-fc treatment with EphA4-fc treatment on chondrocyte maturation. The EphA4-fc fusion protein is a soluble decoy receptor to bind any amounts of the EphA4-binding Efn ligands secreted by chondrocytes. Furthermore, the soluble EphA4-fc could also bind the membrane-associated Efn to trigger the reverse signaling of EphA4. Accordingly, 20,000 cells/well (n=4 well per treatment) were treated with 10 ng/mL EfnA4-fc, 10 ng/mL EphA4/fc, or vehicle control for 7 days in vitro. The number of colonies formed and their colony size were determined using the ImagePro software program, and were compared between control and each treatment group. To determine whether effects were specific for articular chondrocytes, we also determined the effects of EfnA4-fc and EphA4-fc treatments on the number and size of acidic proteoglycan-producing colonies in isolated chondrocytes from

growth plates and from sternum (Fig. 5). Growth plate and sternum chondrocytes were isolated according to established methods.

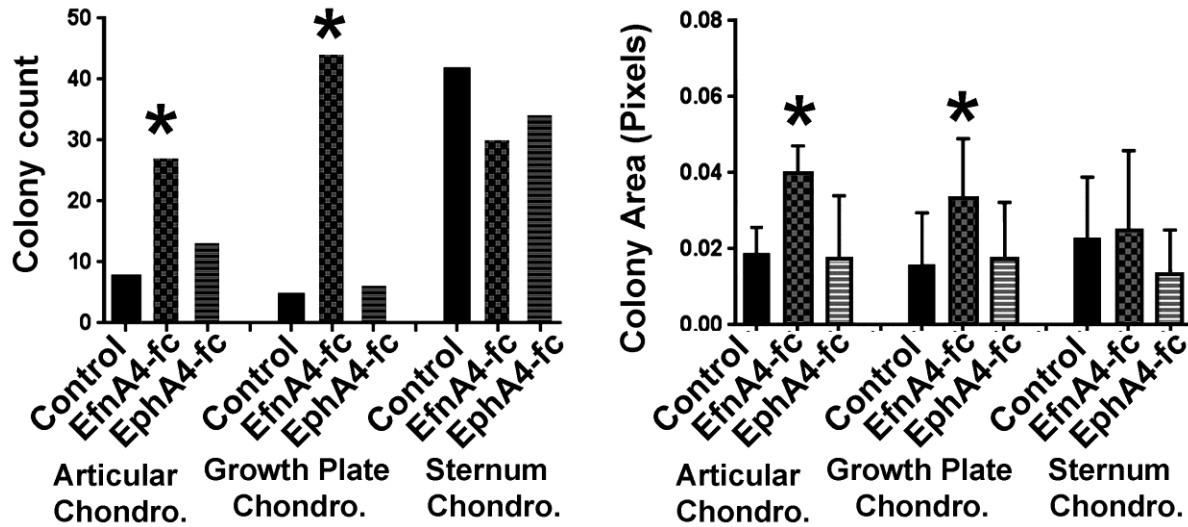


Figure 5. Activation of the forward signaling of EphA4 by treatment with 10 ng/mL EfnA4-fc in articular chondrocytes and growth plate chondrocytes, but not sternum chondrocytes, increased the number and size of acidic proteoglycan-producing colonies after 7 days *in vitro* (* $p < 0.05$, $n = 4$ per group).

Three important points can be made from the results of this preliminary experiment: first, activation of the forward signaling EphA4 signaling by treatment with the soluble EfnA4-fc fusion protein for 7 days significantly increased both the number and the area of acidic proteoglycan-producing colonies in cultured articular chondrocytes as well as in cultured growth plate chondrocytes, supporting our contention that the forward signaling of EphA4 plays an anabolic action in articular chondrocytes. Second, the EfnA4-fc treated did not increase the number or the size of cartilage-producing colonies of sternum chondrocytes, suggesting that the anabolic effects of the forward signaling of EphA4 is specific for only certain chondrocytes, such as articular chondrocytes and growth plate chondrocytes, but had no effect on sternum chondrocytes. Third, the fact that the EphA4-fc treatment had no significant effect on the number or size of the colonies formed from any of the three test chondrocytes suggests either that none of the three test chondrocytes produce or express substantial amounts of EphA4-fc on the cell surface or that the reverse signaling of these EphA4-binding Efn's had no significant regulatory effects on the maturation and/or functional activities of these chondrocytes.

We next assessed whether EfnA4-fc-mediated activation of the EphA4 signaling in articular cartilage would also affect the proliferation of isolated chondrocytes *in vitro*. Cell proliferation was assessed with a commercial MTT assay (Roche Diagnostics Cell Proliferation Kit 1). Very briefly, chondrocytes were plated at 4,000 cells/well in 96 well plates in α -MEM without fetal calf serum, once the chondrocytes reached 70-80% confluence, cells ($n = 6$ per treatment group) were treated with 10 ng/mL EfnA4-fc chimeric fusion protein (a soluble ligand for EphA4 receptor), 10 ng/mL EphA4-fc chimeric fusion protein (a soluble decoy receptor for and a ligand for EphA4-interacting ephrins), or solvent control for 48 hrs. An aliquot of 10 μ L of MTT labeling agent [Roche Diagnostics] was then added directly to each well and incubated for 4 additional hours. An aliquot of 100 μ L of solubilization solution was then added directly to each well and left overnight. The solubilized materials were collected and absorbance at 550-560 nm

was determined on a BioTek Synergy2 plate reader. The absorbance of the control and treated groups was compared to determine the effect of the test agent on cell proliferation. Chondrocytes isolated from three different skeletal sites (i.e., articular cartilage, growth plate cartilage, and sternum cartilage) were used in this study.

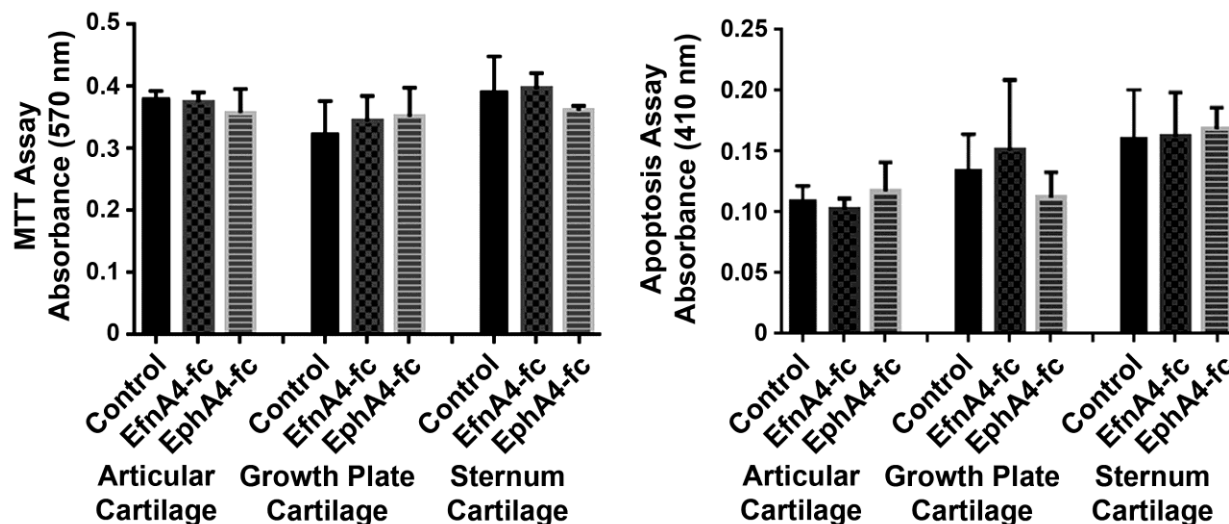


Figure 6. Neither activation of the forward signaling of EphA4 by treatment with 10 ng/mL EfnA4-fc nor blocking EphA4 activation with the 10 ng/mL EphA4-fc had significant effects on the proliferation of chondrocytes isolated from articular cartilage, growth plate cartilage, or sternum cartilage. ($n=6$ per group). None of the treatment group showed statistically significant differences from the corresponding vehicle-treated control group.

As shown in the left panel of Figure 6, neither EfnA4-fc nor EphA4-fc had a significant effect on the proliferation of chondrocytes isolated from the three test cartilages. This is not entirely surprising, since it has been well established that the forward signaling of Eph receptors is potent stimulator of cell differentiation and has no effects on cell proliferation in a number of cell types (Wilkinson, 2014). Thus, this finding, along with our recent findings that the EfnA4-fc treatment significantly enhanced differentiation and maturation of articular chondrocytes, supports the contention that the forward signaling of EphA4 is also a potent stimulator of cell differentiation rather than proliferation in articular chondrocytes.

To evaluate the possibility that activation of the forward signaling of EphA4 in articular chondrocytes could still increase the number of active chondrocytes by suppression their apoptosis, we evaluated whether activation of the EphA4 forward signaling in chondrocytes would alter chondrocyte apoptosis. Briefly, chondrocytes isolated from three different cartilages (i.e., articular cartilage, growth plate cartilage, and sternum cartilage) were plated in 30,000 cells/well in α -MEM supplemented with 10% fetal calf serum in 24-well plate. Once the cells reached ~80% confluence, EfnA4-fc (10 ng/mL), EphA4-fc (10 ng/mL), or solvent vehicle were added and incubated for 7 days with changes of medium every 48 hrs. Cell apoptosis was measured with an apoptosis ELISA kit (Roche) as following. After cell medium was removed, cells were lysed with 500 μ L of lysis buffer for 5 hrs at room temperature. Cell debris was removed by centrifugation, and an aliquot of 20 μ L of each lysate was added into a streptavidin-coated plate. An aliquot of 80 μ L of the pre-mixed immune reagent, which contained anti-DNA-POD, anti-histone-tiotin, and an incubation buffer (Roche), was then added to the lysate. The

immunoreaction was carried out for 2 hrs at room temperature in dark on a shaker revolving at 300 rpm. After washing three times with the incubation buffer, each well was then added with 100 μ L of the ABTS substrate solution, and the plate was incubated at room temperature on a shaker until color developed. Once color appeared, 100 μ L of ABTS STOP solution was added, and the absorbance was read at 405 nm with the BioTek Synergy2 microplate reader. The absorbance indicates the relative levels of cell apoptosis. As shown on the right panel of Figure 6, neither EfnA4-fc nor EphA4-fc had any significant effects on apoptosis of chondrocytes derived from the three test cartilages. Consequently, we conclude that activation (or inhibition) of the forward signaling of EphA4 in chondrocytes also do not affect their apoptosis. Since neither EphA4-fc nor EfnA4-fc affects the proliferation and apoptosis of chondrocytes, especially the articular chondrocytes, we tentatively conclude that the anabolic effects of an activation of the forward signaling of EphA4 in articular cartilage is not due to a change of the cell number but to an activation of their differentiation and maturation.

4. IMPACT:

Injuries to the joint (including articular cartilage, meniscus, and/or ligament), chronic or increased joint loading, or genetic predisposition can lead to lesions in the articular cartilage, which often causes acute and chronic synovial inflammation and osteoarthritis (OA). OA is the most common degenerative joint disease, which afflicts ~50 million people in the US alone with an annual total healthcare cost estimated (in 2011) to be 3 billion dollars. About 12% of all OA are resulted from an acute trauma to the joint, and are referred to as PTOA. While primary OA affects mostly people older than 60 years of age, PTOA can afflict younger and physically active individuals. The military population has a much greater incidence of PTOA than the general population. Accordingly, the rigorous military training and activities are very physically demanding and often impose significant loading impact on the joints of the extremities that can result in injuries to the joint. Repetitive use of the injured joints over the years can irritate and chronically inflame the articular cartilage and synovium causing severe joint pain, swelling, and eventually lead to development of PTOA. Recent advances in body armor have made great strides in decreasing the number of combat-related deaths, but a major consequence is the concurrent increase in complex survival injuries. The majority (>50%) of survival combat injuries are extremity injuries, which includes soft tissue wounds, bone and cartilage defects. In this regard, almost 70% of wounded warriors in the recent Iraq and Afghanistan wars suffered severe extremity injuries and many involved open intra-articular fractures, dislocations, and articular cartilage injuries. These injuries eventually lead to development of PTOA. PTOA is also a major health issue for veterans, as veterans report 50% higher incidence of OA than non-veterans and 43% of VA healthcare users had OA.

There is no effective therapy to prevent or treat PTOA. Patients with mild PTOA are usually treated with medications to manage pain and reduce synovial inflammation. Those with advanced PTOA may require surgeries. Current surgical options include microfracture, structural osteo-articular autografts, or autologous chondrocyte transplantation. Unfortunately, these highly invasive procedures are often unsatisfactory. As a result, the frequent, eventual, end-point is complete joint replacement, which is not a viable option for young or middle-aged service members or veterans, as they often need multiple revision or replacement surgeries over their lifetime. This proposal seeks to develop a novel small molecule-based therapy for prevention

PTOA after joint injuries and for curing advanced PTOA. If this therapy is indeed effective and safe, it could revolutionize the field of PTOA/OA treatment, in that our proposed therapy offers an effective and convenient means that may not only reduce synovial inflammation-induced degradation of the articular cartilage, but may also enhance the regenerative potential of the injured articular cartilage to repair itself. This proposed therapy not only serves as a preventive measure but also as a regenerative treatment for PTOA/OA. This therapy, if successful, would have huge humanitarian benefits as it would greatly improve the quality of life of PTOA patients, not only in the active duty military personnel, in the veteran population, but also in the civilian population. Consequently, if we are successful in developing this novel therapy for OA/PTOA, the impact on the care of military or civilian patients with PTOA/OA will be enormous.

5. CHANGES/PROBLEMS:

There are no administrative issues that would impede performance or progress of this project during the past year.

There is a minor technical issue concerning our ability to obtain sufficient numbers of isolated synoviocytes and/or synovial monocytes for the proposed work. Accordingly, we have developed a reliable method to isolate synoviocytes from the inflamed knee joint. However, the number of purified synoviocytes obtained from a single knee joint is rather limited and is not sufficient for our work. Thus, we need to develop an effective means to expand the isolated cells so that we will be able to have sufficient number of cells. Unfortunately, these cells are highly differentiated and, as such, showed a very poor proliferative capability. We have been having difficulties in expanding the cells in vitro. We are currently trying different culture medium, different lots of fetal bovine sera, and/or addition of different mitogens. If we are still unable to develop an effective means to expand the cell population, we will have to pool primary cells from different mice for our proposed work.

There is also a logistic issue regarding a major change in key personnel that we have to deal with at this time. Accordingly, Dr. Charles Rundle is the co-investigator of this project and is planning to spend 50% of his efforts on this project. However, Dr. Rundle had submitted a BLR&D VA Merit Review proposal last year. This VA Merit Review received approval for funding; the funding of which has just recently been started. The full salary of Dr. Rundle is now being paid through his Merit Review. Accordingly, while Dr. Rundle intends to continue working ~10% on this project in the capacity as an unpaid collaborator, he has recently resigned from this DOD project. It has been very difficult to locate a half-time Ph.D. researcher to work on this project during the remaining two years of this project. Consequently, in order to replace Dr. Rundle on this project, we recently recruited Ms. Virginia Stiffel to work full-time on this project. Ms. Stiffel has a B.S. in Biological Sciences and has more than 30 years of research experience working in places such as University of Chicago, University of Arizona, Loma Linda University. She worked with Dr. Lau, the PI of this project, for the past 10 years. She has also necessary expertise and experience to work on this project. Thus, we request permission to replace Dr. Rundle with Ms. Stiffel. A formal request for this change of key personnel will be submitted to the DOD for approval.

Ms. Kaur, whom we have recently recruited to be the research technician for this project, has informed us that she chose to follow Dr. Rundle to work on Dr. Rundle's VA Merit Review project. Accordingly, she tendered her resignation recently. As a result, we now have to recruit another full-time technician to replace Ms. Kaur in this project. An advertisement for this Research Technician is being prepared and will be post in the very near future. Once a qualified candidate is identified and recruited, we will notify the DOD of the personnel change.

Though the sudden change of personnel may somewhat slow down my immediate progress, we do not believe this issue would have significant long-term impact on our overall progress in this project.

6. PRODUCTS:

No reportable outcomes during this reporting period.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS:

(1) PI:

Name:	Kin-Hing William Lau, Ph.D.
Project Role:	Principal Investigator (PI)
Research identifier (ORCID ID):	0000-0003-1109-5052
Nearest person month worked:	3.6
Contribution to Project:	He plans and coordinates all aspects of the proposed work. He also works with the collaborators and technician to ensure that the proposed studies are successfully executed.
Funding Support:	Full salary support is provided by his VA Research Career Scientist award

(2) Co-Investigator:

Name:	Charles H. Rundle, Ph.D.
Project Role:	Co-Investigator
Research identifier (ORCID ID):	N/A
Nearest person month worked:	6
Contribution to Project:	He is involved in the animal work that involves the intra-articular fracture model and various analyses. He will also assist Dr. Lau in supervising the Research Technician to successfully carrying out the proposed work.
Funding Support:	50% of his salary support was from this DOD award; and the other 50% was from LLVARE.

(3) Co-Investigator:

Name:	Matilda H. Sheng, Ph.D.
Project Role:	Co-Investigator
Research identifier (ORCID ID):	0000-0002-0815-7008
Nearest person month worked:	1.2

Contribution to Project:	She will assist Dr. Lau in the design, interpretation, and execution of the various proposed histological studies. However, this work has not been initiated during the reporting period. Most of the histological work will be performed during year 3 of this project.
Funding Support:	Because her work on this project is not yet required during the past year, no salary support for Dr. Sheng is provided by this DOD project. Dr. Sheng's current full salary support was from the Loma Linda University School of Medicine.

(4) Research Technician:

Name:	Amandeep Kaur, B.S.
Project Role:	Research Technician
Research identifier (ORCID ID):	N/A
Nearest person month worked:	12
Contribution to Project:	Ms. Kaur assisted Dr. Lau and the co-investigators in carrying out the proposed animal and in vitro experimental work under the direct supervision of Dr. Lau. She was assigned experiment work daily by Dr. Lau and she reported to Dr. Lau on the daily basis.
Funding Support:	Her entire salary support was from this DOD award.

8. SPECIAL REPORTING REQUIREMENTS:

Updated Quad chart - See Appendix.

9. APPENDICES:

Updated Quad chart (the following page).

A Novel EphA4-Based Small Molecule-Based Therapeutic Strategy for Prevention and Treatment of Post-Traumatic Osteoarthritis

ERMS/Log Number: OR130346 - To assess feasibility of an EfnA-fc protein-based therapy for PTOA

Award Number: To be assigned



PI: Kin-Hing W. Lau, Ph.D. Org: Loma Linda Veterans Association for Research & Education Award Amount: \$499,960

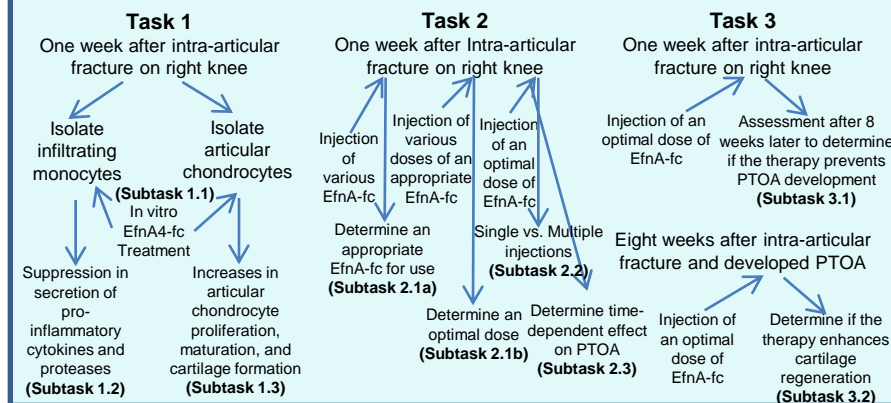
Study Aims

- Aim 1 - To show that activation of EphA4 in synovial monocytes reduces release of pro-inflammatory cytokines and proteinases, but in articular chondrocytes enhances chondrogenesis in vitro.
- Aim 2 - To develop and optimize the EfnA-fc-based strategy involving intra-articular injection of an EfnA-fc into PTOA joint of a mouse.
- Aim 3 - To establish that the EfnA-fc-based therapy is effective in preventing PTOA development and/or treating advanced PTOA in mice.

Tasks/Approach

- Task 1 - Demonstrate in vitro efficacy of the EfnA4-fc approach.
 - Subtask 1.1 - Isolate monocytes and articular chondrocytes for testing (**completed on 28/02/2015**).
 - Subtask 1.2 - Show that EfnA4-fc inhibits activity of infiltrating monocytes.
 - Subtask 1.3 - Show that EfnA4-fc enhances activity of articular chondrocytes.
- Task 2 - Development and optimization of EfnA-fc-based therapy for PTOA.
 - Subtask 2.1 - Identify appropriate form and dosages of EfnA-fc protein for use.
 - Subtask 2.2 - Determine the time-dependent effects of the therapy.
 - Subtask 2.3 - Compare efficacy of single vs. multiple EfnA-fc administration.
- Task 3 - Establish therapeutic efficacy of the EfnA-fc-based strategy.
 - Subtask 3.1 - Show therapeutic efficacy on prevention of PTOA development.
 - Subtask 3.2 - Show therapeutic efficacy on treatment of established PTOA.

Development and Assessment of Feasibility of a Novel EfnA-fc-based Strategy for Prevention and Treatment of PTOA



Accomplishment: Subtask 1.1 is fully completed (28/02/2015).
Subtask 1.3 is fully completed (14/09/2015).

Timeline and Cost

Activities	CY	13	14	15	16
Task 1 - Demonstration of in vitro effects of EfnA4-fc on monocytes and chondrocytes.					
Task 2 - Development and optimization of EfnA-fc-based therapy for PTOA					
Task 3.1- Demonstrate therapeutic efficacy of the therapy on prevention of PTOA.					
Task 3.2- Demonstrate therapeutic efficacy of the therapy on treatment of PTOA.					
Estimated Budget (\$K)*		\$0	\$218	\$233	\$239

* Direct costs + Indirect costs

Updated: (September 14, 2015)

Goals/Milestones

- ❑ **CY14 Goals** - Demonstration of in vitro effects of EfnA4-fc on infiltrating monocytes and chondrocytes; and Development and optimization of EfnA-fc-based therapy for PTOA
- ❑ **CY15 Goal** - To begin work on demonstration of therapeutic efficacy of the therapy on prevention and treatment of PTOA
- ❑ **CY16 Goal** - To complete work on demonstration of therapeutic efficacy of the therapy on prevention and treatment of PTOA

Comments/Challenges/Issues/Concerns

- ❑ Dr. Charles Rundle has recently received approval for his VA Merit Review grant. The effective start date will be September 1, 2015. Thus, his full salary support starting from September 1, 2015, will come entirely from the VA. Although he will continue be a co-investigator of this project, his time effort will be reduced to ~10% from 50%. Accordingly, we will request permission to hire a full-time senior technician to perform the duty left by Dr. Rundle's reduction of efforts, effective September 1, 2015.

Budget Expenditure to Date

- ❑ The total expenditure as of May 31, 2015 is \$104,120.45.